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The effect of a honey based gel and silver sulphadiazine on bacterial infections of in vitro burn wounds

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ABSTRACT

Bacterial contamination remains a constant threat in burn wound care. Topical treatments to combat contaminations have good bactericidal effects but can have detrimental effects for the healing process. Treatments with for example silver can increase healing times. Honey based products can be a good alternative as it is antibacterial and patient-friendly.

We evaluated the bactericidal and cytotoxic effects of a honey based gel and silver sulphadiazine in a human burn wound model with *Pseudomonas aeruginosa*. After adding 10^5 colony forming units of *P. aeruginosa*, topical treatments were applied on the burn wound models. After 2, 12, 24, 28 and 70 h, bacteria were dislodged and counted by plating dilutions. Cytotoxic effects were evaluated histologically in samples of burn wound models treated topically for 3 weeks, without bacteria.

L-Mesitran Soft significantly reduced the bacterial load (5-log reduction) up to 24 h but did not completely eliminate bacteria from the burn wounds. After Flammazine® treatment, only a few colony forming units were observed at all time points. In contrast, re-epithelialization was significantly reduced after application of Flammazine® compared to L-Mesitran Soft or control.

This in vitro model of burn wound infection can be used to evaluate topical treatments. L-Mesitran Soft is a good alternative for treating burn wounds but the slightly lower bactericidal activity in the burn wound model warrants a higher frequency of application.

1. Introduction

Despite significant improvement in the survival of burn patients, infectious complications continue to be a major cause of morbidity and mortality. Burn patients are at high risk of wound infection, sepsis, and ventilator-associated pneumonia because of reduced immune responses. A predominant pathogen cultured from burn wounds in most centres is *Pseudomonas aeruginosa* [1–6]. *P. aeruginosa* is an opportunistic pathogen, which takes advantage of the compromised defence mechanisms in burn patients, namely due to the loss of skin barrier and the suppression of the innate and adaptive immune system [7–12]. *P. aeruginosa* infections in burn patients have been associated with mortality rates of up to 80% [13]. The morbidity associated with *P. aeruginosa* infections is mediated by a combination of several virulence factors such as lipopolysaccharide and haemolysin [14]. Due to its (multi)drug resistance, its abilities to form biofilms and to survive in many hostile environments, *P. aeruginosa* is difficult to eradicate from burn wounds [7,15–17].

Systemic antibiotics can be used but are less favourable because of the low penetration rate into dead tissue and the risk of resistance development. To combat burn wound infections, many different antiseptics are available. Topical treatments have good bactericidal effects but can have detrimental effects on the healing process [18].

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With any wound dressing a balance must be obtained between antimicrobial efficacy and cytotoxicity. The antimicrobial effect of antiseptics is accompanied by cytotoxic side effects [19]. Medical specialists are still challenged in finding the optimal treatment for difficult-to-heal wounds, e.g. ulcers, trauma induced wounds and deep burns. Open wounds are susceptible to invading pathogens such as bacteria and fungi. Therefore, optimal wound healing is dependent on the type of antiseptic medication used in combination with the method of wound closure.

Silver-containg dressings and topical silver agents represent one of the current standards for the treatment of superficial and deep dermal burns, because silver is an effective, broad-spectrum antimicrobial agent [20–23]. Resistance to silver can occur in some bacterial species albeit only to a low level [22,24]. Various reports however suggest that silver-sulphadiazine delays wound healing by enhancing the proinflammatory cytokines and that its use may lead to poor scarring [25–27]. Despite advantages as reduced use of systemic antibiotics and slow release of silver ions in order to promote healing, cellular toxicity remains a problem. Silver sulphadiazine is only to be used for a limited period in the treatment of burns rather than for the entire treatment period. Silver sulphadiazine was found to be cytotoxic for human skin and skin subsitutes [18,28]. Topical silver might delay wound healing, because of its toxicity to keratinocytes [29]. However, whether silver is detrimental for keratinocytes is still under debate [30]. Based on the recent and thorough systematic review and meta-analysis of 14 RCT's by Aziz et al. it can be concluded that the evidence for infection prevention as well as inducing of healing is contradictory and limited due to the small number of well-designed and well conducted comparative studies [31]. Although in general the antimicrobial effect of silver is not under debate, no conclusion can be reached based on current literature as to the right dosage of silver in vivo in wound management [32].

Another approach in this area is the development of dressings with a controlled release of antibiotics or biocides. This strategy allows high levels of antibiotic drugs to specifically reach the wound area and avoids the disadvantage of current antimicrobial creams and ointments that need frequent dressing changes. Although promising results have appeared recently in the literature [33], these data are still from an experimental setup and no clinical data have been presented so far. Clinical results with biocide impregnated dressings indicate a need for improvement on infection prevention and clinical outcome (healing time) [34,35].

A good alternative to silver based dressings might be honey, which has no adverse effects on wound healing. Honey has been used as a topical treatment for infection throughout history [36]. Honey may improve healing times in mild to moderate superficial and partial thickness burns compared to some conventional dressings [29,37]. Honey also appears to be effective in wound deodorizing and debridement; it has an anti-inflammatory effect and can stimulate tissue growth [38]. The antimicrobial properties of honey are mediated by hydrogen peroxide, high sugar content, low pH, methylglyoxal and bee defensin-1 [39]. Activation of enzymes in the host and the osmotic ability of honey both stimulate autolysis of necrotic tissue [38]. Honey promotes the formation of healthy granulation tissue [38], optimizes epithelialization [38] and angiogenesis [40], and oxygen uptake is induced. Hydrogen peroxide is produced by glucose oxidase in honey and stimulates the growth of fibroblasts and epithelial cells [41]. This all contributes to the promotion of tissue regeneration.

Because honey in its pure form is not patient-friendly (e.g. it runs off the body when warm), L-Mesitran Soft was developed. This anti-bacterial gel can be used to manage a variety of (chronic) wounds in a wide range of stages of the healing process. The antibacterial effect of honey and L-Mesitran Soft has been shown in vitro [27,29,39,42] but not yet in a burn wound model based on human skin. Also, the effects of L-Mesitran Soft on burn wound healing have not been shown in vitro.

In this study we investigated the bactericidal effect of L-Mesitran Soft on *P. aeruginosa* in a human burn wound model. For comparison, we used a silver sulphadiazine cream (Flammazine®), which is being used in Dutch burn centres. In addition, we tested the effect of these topical treatments on re-epithelialization.

2. Materials and methods

2.1. Materials

L-Mesitran Soft (Triticum Group, Maastricht, The Netherlands) is a gel that contains 40% honey, hypoallergenic lanolin, vitamin C, vitamin E and polyethylene glycol 4000.

2.2. Burn wound model

Human skin was obtained from 13 healthy donors undergoing dermolipectomy, or from 11 deceased donors via the Euro Tissue Bank (Beverwijk, The Netherlands), after obtaining consent according to institutional guidelines. The burn wound model was performed as described by Coolen et al. [43]. A split-thickness skin graft (0.5 mm thick) was burned for 10 s with a copper device (2 × 10 mm) heated to 95 °C. Skin samples were cultured air-exposed at 37 °C with 5% CO_2 for up to 3 weeks. Medium consisted of Dulbecco's modified Eagle's medium/Ham's F12 (3:1) (Invitrogen, Paisley, UK), 2% fetal calf serum (Hyclone, Logan, UT), 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin, 10 μM of L-carnitine, 10 mM of L-serine, 1 mM of DL-α-tocopherol, 130 μg/mL of ascorbic acid, a lipid supplement (containing 25 μM palmitic acid, 15 μM linoleic acid, 7 μM arachidonic acid, and 24 μM bovine serum albumin) (all Sigma–Aldrich, St. Louis, MO), and penicillin/streptomycin (100 IU/mL penicillin, 100 mg/mL streptomycin; Invitrogen) and was refreshed twice a week.

2.3. Bactericidal activity in the burn wound model

P. aeruginosa (strain PAO1) was routinely cultured on Luria Broth (LB) agar at 37 °C. Colonies were suspended in LB medium and cultured for 4 h at 37 °C, 250 rpm. Bacteria were spun down, re-suspended in PBS and diluted in PBS until the desired optical density at 650 nm was obtained.

Thirty minutes after inflicting burn wounds (see burn wound model above), the burned areas were inoculated with 10 μl of an overnight LB culture containing approximately

10^5 colony forming units (CFU) of PAO1. After 45 min of incubation, 100 μl of L-Mesitran Soft or 1% silver sulphadiazine cream (Flammazine®, Solvay Pharma, Weesp, The Netherlands) was applied on the burned and inoculated areas. Untreated inoculated skin samples served as controls. The samples were cultured at the air–liquid interface in medium as described above at 37 °C with 5% CO_2. Antibiotic concentrations in the culture medium were adjusted to 25 IU/ml penicillin, 25 mg/ml streptomycin (Invitrogen, Paisley, UK). After 2, 12, 24, 48 and 70 h of incubation, skin samples were placed in cryovials containing 1 ml of PBS solution and a 7 mm (diameter) metal bead. Cryovials were placed in a TissueLyser LT (Qiagen, The Netherlands) for 4 min at 15 Hz. Bacterial suspensions were serially diluted and plated on LB agar plates in triplicate. After overnight incubation at 37 °C, colonies were counted. The antibacterial effect of the treatment was quantified by the log reduction, $\log(N_T/N_C)$, where N_T is the number of viable cells after treatment and N_C the number of viable cells in control samples. Skin samples were fixed in Kryofix (50% ethanol, 3% PEG300). The experiment was performed 6 times with skin from different donors.

2.4. *Effect of topical treatments on in vitro wound healing*

Thirty minutes after inflicting burn wounds (see burn wound model above), the burned areas were topically treated once with 50 μl L-Mesitran Soft (19 donors) or Flammazine® (12 donors). As controls, burned areas were left untreated and air-exposed (12 donors) or were treated with 50 μl Vaseline (3 donors) or Cetomacrogol (5 donors). Vaseline and Cetomacrogol are frequently used to formulate creams and were used to determine the effect of topical occlusion. Cetomacrogol is the base of Flammazine® without active components. For every donor the experiments were carried out in duplicate. Skin samples were kept in culture for three weeks; culture medium was refreshed twice a week. To evaluate proliferation of keratinocytes in the burn wound model, the skin samples were incubated with 20 mM of bromodeoxyuridine (BrdU, Sigma–Aldrich, St. Louis, MO, USA) for 24 h before fixation. Skin samples were fixed in Kryofix.

2.5. *Histology and immunohistochemistry*

Burn wound samples were processed for paraffin embedding. Sections (4 μm) were deparaffinised and rehydrated for haematoxylin and eosin (H&E) staining, using standard techniques. The newly formed epidermis and total wound area were measured with digital image analysis (NIS Elements Ar software, Nikon, Leiderdorp, The Netherlands).

To visualize bacteria, slides were stained with acridine orange stain (3,6-bis(dimethylamino)acridine) for 2 min and rinsed with water [21]. Slides were viewed using fluorescence microscopy.

To evaluate proliferation of keratinocytes in the burn wound model, the incorporation of BrdU was examined. Sections (4 μm) were deparaffinised, rehydrated, and treated with H_2O_2 (1% in PBS; Sigma–Aldrich) for 20 min. The cellular DNA was denatured in 2 N HCl for 30 min and neutralized with 0.1 M Borax (Sigma–Aldrich). The sections were incubated with monoclonal anti-BrdU antibody (1:100 in 1% BSA; MP Biomedicals, Illkirch, France) for 1 h, followed by incubation with poly-HRP anti-mouse (Powervision, Immunologic, Duiven, The Netherlands) for 30 min at room temperature. Peroxidase activity was detected with 3,3′-diaminobenzidine substrate. All sections were counterstained with haematoxylin. Numbers of proliferating cells were counted in the newly formed epidermis.

2.6. *Statistics*

Statistical analysis was performed with SPSS (Version 16.0 for MS Windows, SPSS Inc, Chicago, IL). The Wilcoxon matched-pairs signed rank test (WMP) and the Mann–Whitney *U* test (MWU) were used to determine significant differences between the groups.

3. Results

3.1. *In vitro model of burn wound infection*

Infection of the burn wound model with 10^5 CFU of PAO1 resulted in biofilm-like growth on the epidermis, which expanded to the edges of the epidermis. At that location, bacteria translocated into the dermis and proliferated in between the dermis and the epidermis (Fig. 1). The epidermis was clearly affected by PAO1, leading to detachment and destruction of the epidermis. After 3 days of culture, the skin sample was completely colonized. Longer incubation times were not possible because of growth of *P. aeruginosa* in the culture medium of untreated controls.

3.2. *Bactericidal activity in the burn wound model*

Bacterial load was measured by plate counting inoculated burn wound samples (6 donors) after 0, 12, 24, 48 and 70 h (Fig. 2A). In untreated control samples, the number of bacteria quickly increased to 10^9 CFU/ml after 24 h. L-Mesitran Soft greatly reduced the bacterial load significantly up to 24 h but did not completely eliminate bacteria from the burn wounds. Bacterial loads in L-Mesitran Soft treated samples increased to an average of 10^7 CFU/ml after 48 and 70 h, which was still lower than untreated controls. After Flammazine® treatment, only a few CFU were observed.

Variation in bacterial load was high after L-Mesitran Soft treatment, e.g. 10^8 versus 0 CFU/ml, which skews the results. Therefore, we calculated the log reductions using the CFU values per donor. For all timepoints, 6-log reductions or more were observed for both L-Mesitran Soft and Flammazine® (Fig. 2B). Log reduction after L-Mesitran Soft treatment was lower compared to Flammazine® after 48 and 70 h.

Acridine orange staining was used to evaluate the effects of topical treatments on bacterial numbers at the microscopic level. Thick bacterial layers penetrating the dermis underneath the epidermis after 48–70 h of culture were only observed in untreated control samples (Fig. 1A and B). In untreated control samples, detachment and degradation of the epidermis could be seen after 48–70 h of culture (Fig. 1A and B). This was not observed in the L-Mesitran Soft and Flammazine® treated samples. After 70 h of incubation, bacteria were

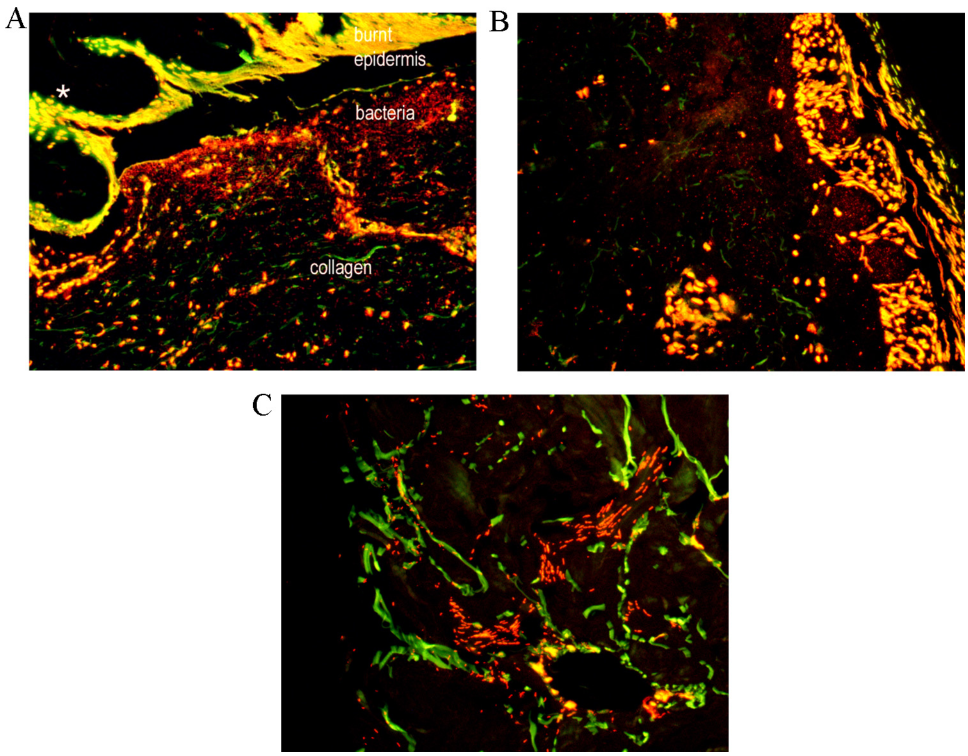

Fig. 1 – Acridine orange stain of the inoculated skin samples after 3 days of culture. (A) Bacteria (red) can be seen in the burn wound area. Nuclei stain orange/yellow while collagen, burnt epidermis and stratum corneum stain green. Asterisk indicates detachment of epidermis due to *P. aeruginosa* and not due to burning. (B) Thick bacterial layers in the dermis underneath the epidermis after 48–70 h of culture as observed in untreated control samples. (C) Bacteria in the reticular dermis after 70 h of culture. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

observed at the reticular side of the dermis (which was in contact with the medium) in the untreated controls (Fig. 1C) and in some cases of the L-Mesitran Soft and Flammazine® treated samples. The number of bacteria observed with acridine orange staining was in line with the CFU results.

3.3. *Topical treatments and in vitro wound healing*

Topical antibacterial treatment of wounds may affect healing. To evaluate the effect of the treatments on re-epithelialization, burn wound models were treated topically or left air-exposed and cultured for three weeks (Table 1). Cetomacrogol is the base of Flammazine® and was included as control treatment. Sizes of the wounds and the newly formed epidermis were measured in H&E or acridine orange stained sections. Wound sizes did not differ between treatments or experiments. All topical treatments resulted in a reduced outgrowth compared to the untreated air-exposed control. However, application of Flammazine® resulted in significantly less re-epithelialization compared to L-Mesitran Soft or Cetomacrogol.

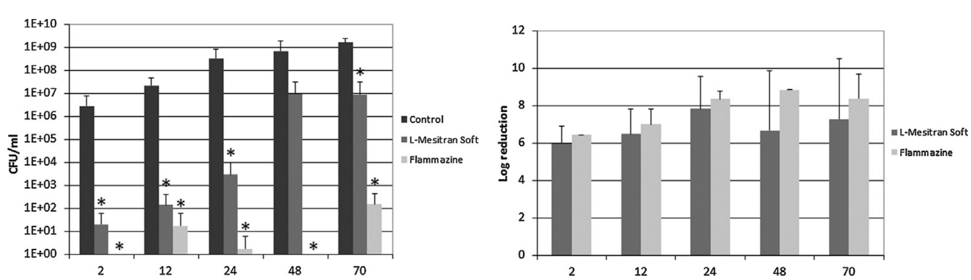

Fig. 2 – Bactericidal activity in the burn wound model. (A) CFU from burn wound samples at different time points after different topical treatments. Shown is the average of samples from 6 donors ± standard deviation. *Significant difference from the untreated control (MWU, $P < 0.05$). (B) Log reduction at different time points after different topical treatments. Log reduction was calculated by $\log(N_T/N_C)$, where N_T is the number of viable cells after treatment and N_C the number of viable cells in control samples.

Table 1 – Re-epithelialization in the burn wound model treated topically and controls. Shown are average values ± standard devation. Significant differences compared to the air-exposed control (*) or Flammazine® (#) are indicated (WMP, $P < 0.05$).

	Topical treatments	Re-epithelialization (mm)	Number of donors	Number of BrdU+ keratinocytes (mm)	Number of donors
Controls	Air-exposed	$496 \pm 221^{\#}$	12	57 ± 16	3
	Vaseline	322 ± 191	3	$4 \pm 7^{*}$	3
	Cetomacrogol	324 ± 141	5	$12 \pm 20^{*}$	4
	L-Mesitran Soft	$335 \pm 151^{*,\#}$	19	$7 \pm 9^{*}$	10
	Flammazine®	$172 \pm 69^{*}$	12	$4 \pm 9^{*}$	9

In addition, the effect on proliferation of keratinocytes was determined by using BrdU incorporation (Fig. 3). In samples after 21 days of culture, the numbers of proliferating cells ($BrdU^+$) were quantified in the newly formed epidermis (Table 1). Any treatment resulted in reduced proliferation, which is in line with the results on re-epithelialization.

4. Discussion

We investigated bactericidal activity and effect on re-epithelialization of both L-Mesitran Soft and Flammazine® in our burn wound model. Bactericidal activity of L-Mesitran Soft in the burn wound model seems to be less effective in comparison with Flammazine®. However, the viscosity of L-Mesitran Soft is lower than that of Flammazine®, it spreads more easily on skin and is therefore easier diluted. This might have resulted in a lower concentration and activity. Application of L-Mesitran Soft on a daily basis or in a gauze might improve the results even further. Flammazine® has good diffusing ability [18], the bactericidal effect reaches a larger area, and does not need a daily application of the treatment.

Longer incubations of the burn wound model with bacteria were not performed as this increases the risk that bacteria move to the medium or to the reticular side of the dermis, where topical treatments are less effective.

Air exposure of skin samples during culturing is required to preserve viable cells in the burn wound model [43]. The results on re-epithelialization and proliferation underscore this. The best results were obtained with the air-exposed untreated control. Good results were obtained with L-Mesitran Soft on re-epithelialization, the low viscosity probably contributed to a good air-permeability. Although diffusion in skin of antimicrobial components from Flammazine® is good, the high viscosity results in low air-permeability. This combination can be detrimental for cell or skin culture. Detrimental changes in tissue histology, such as vacuole formation, condensed nuclei and separation of the epidermis from the dermal matrix were directly related to the cytotoxicity of the treatment with Flammazine® [18]. In many cases the epidermis detached from the dermis around the wound margins, mostly in samples treated with Flammazine® or Cetomacrogol. Outgrowth of the newly formed epidermis could not be measured in those samples. In samples treated with L-Mesitran Soft, detachment of the epidermis was observed only occasionally.

Proliferation of keratinocytes in the burn wound model slowly declines when cultured for a longer period. After 7 days of culture nearly 100% of the basal keratinocytes in the neo-epidermis should be proliferating [43]. Proliferation slowly declined from 60 after 1 week to 37 BrdU-positive keratinocytes/mm after 3 weeks of culture [44]. In our air-exposed controls, the keratinocytes showed a relatively high proliferation of 57 BrdU-positive cells/mm.

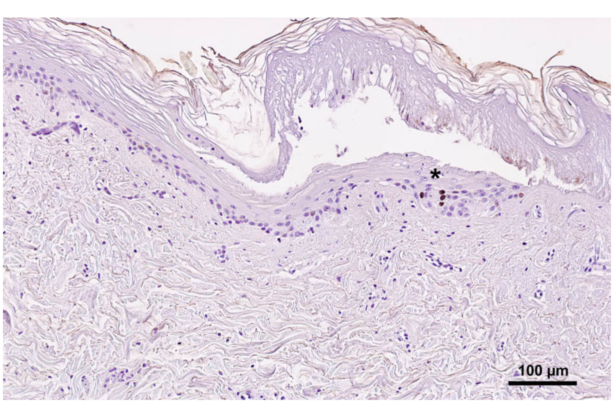

Fig. 3 – The newly formed epidermis with visualization of proliferating cells. Asterisk indicates BrdU incorporation: cells with brown stained nuclei. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

5. Conclusion

We have shown that this in vitro model of burn wound infection can be used to study the effects of topical treatments. Application of L-Mesitran Soft resulted in significantly more re-epithelialization compared to Flammazine®. The slightly lower bactericidal activity in the burn wound model warrants a higher frequency of application of L-Mesitran Soft.

Disclosure

This work was financially supported by the Triticum Group, Maastricht, The Netherlands.

Conflict of interest

None of the authors have any financial and personal relationships with other people or organizations that could inappropriately influence (bias) this work.

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